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Downregulated regulatory T cell function is associated with increased peptic ulcer in *Helicobacter pylori*-infection

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ABSTRACT

Background: *Helicobacter pylori* (*H. pylori*) chronically colonizes gastric/duodenal mucosa and induces gastroduodenal disease such as gastritis and peptic ulcer and induces vigorous innate and specific immune responses; however, the infection is not removed, a state of chronic active gastritis persists for life if untreated. The objective of this study was to determine the number of regulatory T cells (Tregs) in gastric mucosa of patients with gastritis and peptic ulcer and determined the relationship between main virulence factor of *H. pylori* and Tregs.

Methods and materials: A total of 89 patients with gastritis, 63 patients with peptic ulcer and 40 healthy, *H. pylori*-negative subjects were enrolled in this study. Expression of CD4 and Foxp3 was determined by immunohistochemistry. Antrum biopsy was obtained for detection of *H. pylori*, bacterial virulence factors and histopathological assessments. TGF-β1, IL-10 and FOXP3 expressions were determined by real-time polymerase chain reaction (qPCR).

Results: The numbers of $CD4^+$ and $Foxp3^+$ T cells as well as the expression of IL-10, TGF-β1, FOXP3, INF-γ and IL-17A in infected patients were significantly higher than the ones in uninfected patients. Also, the number of $CD4^+$ T cells was independent on the vacuolating cytotoxin A (vacA) and outer inflammatory protein A (oipA), but it was positively correlated with cytotoxin-associated gene A (cagA). Instead, the number of $Foxp3^+$ T cells was dependent on the vacA and oipA, but it was independent on cagA. The number of $Foxp3^+$ T cells and the expression of IL-10, TGF-β1 and FOXP3 in infected patients with gastritis were significantly higher than the ones in infected patients with peptic ulcer. Moreover, the number of $CD4^+$ T cells and the expression of IL-17A and INF-γ was the lowest in the gastritis patients, however, increased progressively in the peptic ulcer patients. Additionally, the numbers of $CD4^+$ and $Foxp3^+$ T cells as well as the expression of IL-10, TGF-β1, FOXP3 and INF-γ were positively correlated with the degree of *H. pylori* density and chronic inflammation.

Conclusion: Tregs are positively associated with vacA alleles and oipA status of *H. pylori* and histological grade but negatively associated with peptic ulcer disease.

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1. Introduction

Helicobacter pylori (*H. pylori*) is a helix shaped, Gram-negative, microaerophilic, flagellated bacteria. It is one of the highly abundant human pathogens that infects approximately 50% of the world population [1]. Infection with *H. pylori* is usually acquired in early childhood but in the absence of treatment persists for life [2]. Although the vast majority (>80%) of infected individuals are asymptomatic, about 10–20% will develop peptic ulcers (PUD), 1 to 2% gastric cancer [3] and 0.1% mucosa-associated lymphoid tissue (MALT) lymphoma [4]. These different clinical outcomes have been attributed to the interplay of several factors, including virulence factors of *H. pylori*, host genetic susceptibility, local innate and adaptive immune responses, as well as environmental conditions (e.g. smoking, malnutrition, high salt intake, vitamin and antioxidants deficiency) [5]. Morbidity and mortality associated with peptic ulcer diseases (gastric and duodenal ulcers) have had significant threats to the world's population over the past two centuries [6]. Although the underlying mechanism of PUD development due to *H. pylori* infection is not fully understood, untreated chronic inflammation in patients infected with *H. pylori* is thought to contribute to the continued damage to the stomach epithelium [7]. *H. pylori* infection causes severe local inflammation in the gastric mucosa [8]. For instance, increased $CD3^{+}CD4^{+}$ T cells in gastric lamina propria (LP) of patients infected with *H. pylori* may play an important role in the pathogenesis of persistent infection [9]. During *H. pylor*i infection, the frequency of $CD4^{+}$ T cells in the lamina propria of stomach with a memory phenotype are increased and polarized to Th1/Th17 phenotypes, but these T cells are hyporesponsive against this bacterium [10]. Because this hyporesponsiveness contributes to chronic infection, there have been targeted efforts to understand the mechanisms employed by *H. pylori* to downregulate T cell responses. *H. pylori* also manipulates T cell function by eliciting Tregs which are frequently found in these patients [11]. Tregs are usually characterized by expression of CD4, high levels of CD25 ($CD25^{high}$), and the transcription factor FOXP3, which play crucial role in maintaining self-tolerance and control of autoimmune diseases in both mice and humans [8]. Tregs can be divided into two subgroups, natural Tregs (nTreg) and inducible Tregs (iTreg), based on their maturation site [12]. The natural Tregs are developed during normal T-cell maturation in the thymus and enter peripheral tissues where they suppress the activation of self-reactive T cells [12]. The iTregs are directly developed in the peripheral lymphoid organs from naive T cells after antigen priming. Tregs mediate their inhibitory activities by production of immunosuppressive cytokines such as IL-10, TGF-β and IL-35 [12] or by cell-cell interactions [13]. Recent studies have shown that Tregs suppress the immune response to *H. pylori* infection [8,14,15]. There is growing evidence indicating that the failure of the host to eradicate *H. pylori* may be due to the ability of the pathogen to evade T cell immunity by inducing Tregs. Recently, *H. pylori*-specific Tregs were shown to suppress memory T-cell responses in infected individuals [16,17]. Studies in mice infected with *H. pylori* have shown that depletion of Tregs leads to increased gastric inflammation and reduced colonization of *H. pylori* [14]. These findings suggest that Tregs contribute to the persistence of *H. pylori* colonization in gastric mucosa, which may then lead to development of peptic ulcer diseases. However, the relationship between Tregs and the pathogenesis of peptic ulcer remain unclear. Therefore, the aim of this study was to investigate the relationship between the frequency of Tregs and *H. pylori* virulence factors in a cohort of Iranian adult patients. In addition, we examined Tregs frequency with the disease clinical outcomes in this population.

2. Materials and methods

2.1. Study population

A total of 192 patients were examined by upper gastroduodenal endoscopy at Hajar General Hospital in Shahrekord, Iran. Among them, 128 patients who had gastric antral biopsy were considered for histological diagnosis for this retrospective study. After medical chart review, patients with chronic heart, kidney, lung or liver diseases, having history of gastric surgery or anti-*H. pylori* eradication therapy, as well as patients taking non-steroidal anti-inflammatory drugs within one week prior to endoscopy were excluded from this study. The remaining patients were classified into three groups according to the results of endoscopic and histological examinations; a) 55 gastritis (G; 28 females, 27 males), b) 47 peptic ulcer diseases (PUD; 16 females, 31 males), and c) 26 uninfected subjects (G; 17 females, 9 males). The mean age was 46.38 ± 14.8 years among G subjects, 48.43 ± 15.6 years among PUD cases and 47.17 ± 19.0 years among uninfected individuals.

2.2. Patient sampling

The appropriate Institutional Ethic Committees of The Tehran University of Medial Sciences and Shahrekord University of Medical Sciences approved the human studies. All study participants gave written informed consent to participate in this study. Biopsy specimens were obtained from the antral region of the stomach. Four gastric mucosal biopsy specimens were obtained from each patient. Two specimens were used for DNA and RNA extraction and one specimen was used for histopathological studies. One antral specimen was directly placed into a medium containing urea to perform the rapid urease test (RUT). The results were recorded within 24 h. A positive RUT was indicated when the color changed from yellow to pink. *H. pylori*-infection was determined by the rapid urease test, PCR (*16srRNA* and *glmM*) and histological examination. Patients were classified as *H. pylori*-infected only if the four tests were positive.

2.3. Microscopic examination and immunohistochemistry

Two biopsy specimens were taken from the antrum for histopathological evaluation. Sections of biopsy specimens were embedded in 10% buffered formalin, and then embedded in paraffin, cut in sequential 4 μm sections and subsequently stained with hematoxylin and eosin (H&E) for grading and evaluating the severity of gastritis. The specimens were also subjected to modified Giemsa staining for visualization of *H. pylori* using light microscopy. The colonization of *H. pylori* on the gastric epithelium was graded on a 3-point scale of severe colonization (the presence of large groups of organisms on the surface and upper pits of more than two-thirds of the mucosal surface), mild colonization (individual organisms, covering less than one-third of the mucosal surface) and moderate colonization (between these two). We defined an ulcer as a circumscribed mucosal break (>5 mm in diameter, with apparent depth) in the stomach or duodenum, covered with exudates. The histological severity of gastritis was blindly graded from normal to severe based on the degree of mononuclear cell (MNC) and polymorphonuclear leukocyte (PMN) infiltration according to the Updated Sydney system [18] on a four-point scale: 0, no; 1, mild; 2, moderate; and 3, severe changes. For immunohistochemical analysis, 4-μm serial sections were made and spread on poly-L-lysine-coated slides. Paraffin sections were dried in a 70 °C oven for overnight, deparaffinized in three changes of xylene and hydrated using a series of alcohols (100%, 100%, 80% and 70%). Antigen retrieval was performed routinely by immersing the sections in

citrate buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0) in a pressure cooker by autoclaving for 20 min. The sections were then incubated with protein block (Abcam, England) for 60 min to block nonspecific background staining. Subsequently, Rabbit anti-human CD4 antibody (ab133616, Abcam, UK) at a 1:450 dilution and Rabbit anti-human FOXP3 antibody (ab99963, Abcam, UK) at a 1:350 dilution were applied, respectively, to the sections that were latter incubated overnight in a humidified chamber at 4 °C. On the second day, endogenous peroxidase activity was blocked with 3% H2O2 in TBS for 15 min. Afterwards, Biotinylated goat anti-rabbit and mouse IgG (ab93697, Abcam, UK) were applied and the sections were incubated for 1 h at room temperature. Then, applying Streptavidin Peroxidase Plus, the sections were incubated for 10 min at room temperature. Afterwards Applying DAB (ab94665, Abcam, UK) to tissue, the sections were incubated for 10 min. Sections were counterstained for 1 min with Meyer's hematoxylin and then mounted. Human hodgkin's lymphoma tissue was used as a positive control for Foxp3. Additional sections were processed without primary antibody as a negative control. The number of $CD4^+$ and Tregs was calculated by counting positive lymphocytes throughout the entire area of tissue section at 10 high power fields. Results were expressed as the mean value and interquartile range of all tested patients in each group.

2.4. H. Pylori detection and virulence genotyping

Detection of *H. pylori* and virulence genotyping was performed by polymerase chain reaction (PCR). Primer sequences and PCR conditions reported by salimzadeh et al. [19].

2.5. Real-time PCR

Total RNA from the biopsy samples was extracted using a TRIzol® Plus RNA Purification Kit according to the supplier's instructions. Complementary DNA (cDNA) was synthesized using reverse transcriptase (RT) using the First Strand cDNA Synthesis Kit (Fermentas Life Sciences, cat- K1622). For cDNA synthesis 2.5 μg of pure RNA was used as template. Using the TaqMan RT-PCR system, amplification of IL-10, TGF-β1, FOXP3 and β-actin cDNA was performed in a Rotorgene 3000 (Corbett Research). The real time-PCR reactions were performed in a total volume of 20 μl containing 5.75 μl of nuclease-free H2O, 3 μl of synthesized cDNA solution, 10 μl of 2x Rotor-Gene Probe PCR Master Mix (Qiagen, Germany), 0.5 μl of each primer (10 pM) and 0.25 μl of the TaqMan probe (10 pM). Negative controls for real time-PCR amplification were prepared by omitting the cDNA sample from the reaction mixture. Thermal cycling was initiated with a first denaturation step at 95 °C for 10 min and followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s. The primer and probe sequences for IL-10, TGF-β1, FOXP3, INF-γ, IL-17A and β-actin cDNA have been previously published [4,20–23]. Expression of IL-10, TGF-β1, INF-γ, IL-17A and FOXP3 mRNA relative to β-actin mRNA were determined using the $2^{-\Delta\Delta Ct}$ method.

2.6. Statistical analysis

Experiments were performed in duplicate, and all data were shown as mean ± SEM. The normal distribution of data was confirmed by Shapiro-Wilk normality test. Continuous ratio scale data were evaluated by unpaired Student *t*-test (for comparison between two samples) or by ANOVA (for multiple comparisons) and Turkey post hoc analysis, which compares infected groups to a specific control group by GraphPad Prism software version 5 (GraphPad Software, La Jolla, CA, USA). Categorical data (between virulence factors and type of disease) were presented as frequencies. Comparisons were made using the Chi-square or Fisher exact test if < 5 samples were in one group. Statistical significance was assumed if a *P*-value was less than 0.05.

3. Results

3.1. Relation of $CD4^+$, $Foxp3^+$ T cells and cytokines expression with gastroduodenal diseases

The presence of $CD4^+$ and $Foxp3^+$ T cells in the gastric mucosa of gastroduodenal diseases were determined in two consecutive sections. The expression of CD4 was found on the surface of lymphocytes, whereas the expression of Foxp3 was seen in the nucleus of lymphocytes (Fig. 1A–F). We observed infected patients with peptic ulcer had significantly higher number of $CD4^+$ cells than infected patients with gastritis and uninfected patients (Fig. 2A). We also found that the number of $Foxp3^+$ T cells in infected patients with gastritis was significantly higher than infected patients with peptic ulcer and uninfected patients (Fig. 2B). To determine the immunomodulatory effects of *H. pylori* infection at the mucosal surfaces, we analyzed expression of cytokines (e.g. IL-10, TGF-β1, IFN-γ and IL-17A) and FOXP3 in the gastric tissues by real time-PCR. The relative mRNA levels of IL-10, TGF-β1 and FOXP3 were significantly elevated in infected patients with gastritis compared to infected patients with peptic ulcer and uninfected patients. The fold changes in IL-10, TGF-β1 and FOXP3 expression levels in the infected patients with gastritis compared to uninfected individuals were 2.6, 3.6 and 3.47 respectively. In addition, we noted higher expression of IL-10, TGF-β1 and FOXP3 mRNA in mucosal tissues obtained from peptic ulcer patients compared to uninfected individuals however their expression levels were lower compared with gastritis patients. Infected patients with gastritis had higher expression of mRNA for IL-10, TGF- β1 and FOXP3 compared with peptic ulcer which enumerated by 1.73, 1.33 and 1.36 folds respectively (Fig. 2C–E). Furthermore, we measured expression levels of INF-γ and IL-17A in mucosal biopsies. As indicated in Fig. 2F–G, we observed significantly higher expression of these cytokines in infected patients with peptic ulcer compared to infected patients with gastritis and uninfected patients. The fold changes in INF-γ and IL-17A expression in infected patients with peptic ulcer compared with uninfected patients were 5.1 and 4.04 respectively. However, the fold changes in INF-γ and IL-17A expression between the infected patients with peptic ulcer and infected patients with gastritis were respectively 2.18 and 1.9 respectively (Fig. 2F–G).

3.2. Relation of $CD4^+$, $Foxp3^+$ T cells and cytokines expression with allelic variants of vacA

We aimed to determine the role of *H. pylori* toxin, the vacuolating cytotoxin (*vacA*) in immunological changes observed in the mucosal tissues of different patients. We found no correlation between enumerated $CD4^+$ T cells in *H. pylori*-infected patients and the vacA status of the pathogen (Fig. 3A; $P = 0.79$). However, the number of $Foxp3^+$ T cells was dependent on the different allelic variants of vacA (Fig. 3B; $P = 0.007$). In addition, we observed that mucosal IL-10, INF-γ and IL-17A mRNA levels were independent on the vacA status and no significant difference was found in the expression of IL-10, INF-γ and IL-17A when comparisons were done between infected patients with different allelic variants of vacA (Fig. 3C, F and 3G). Interestingly, mucosal TGF-β1 and FOXP3 mRNA levels were associated with the different allelic variants of vacA (Fig. 3D and E; $P < 0.0001$).

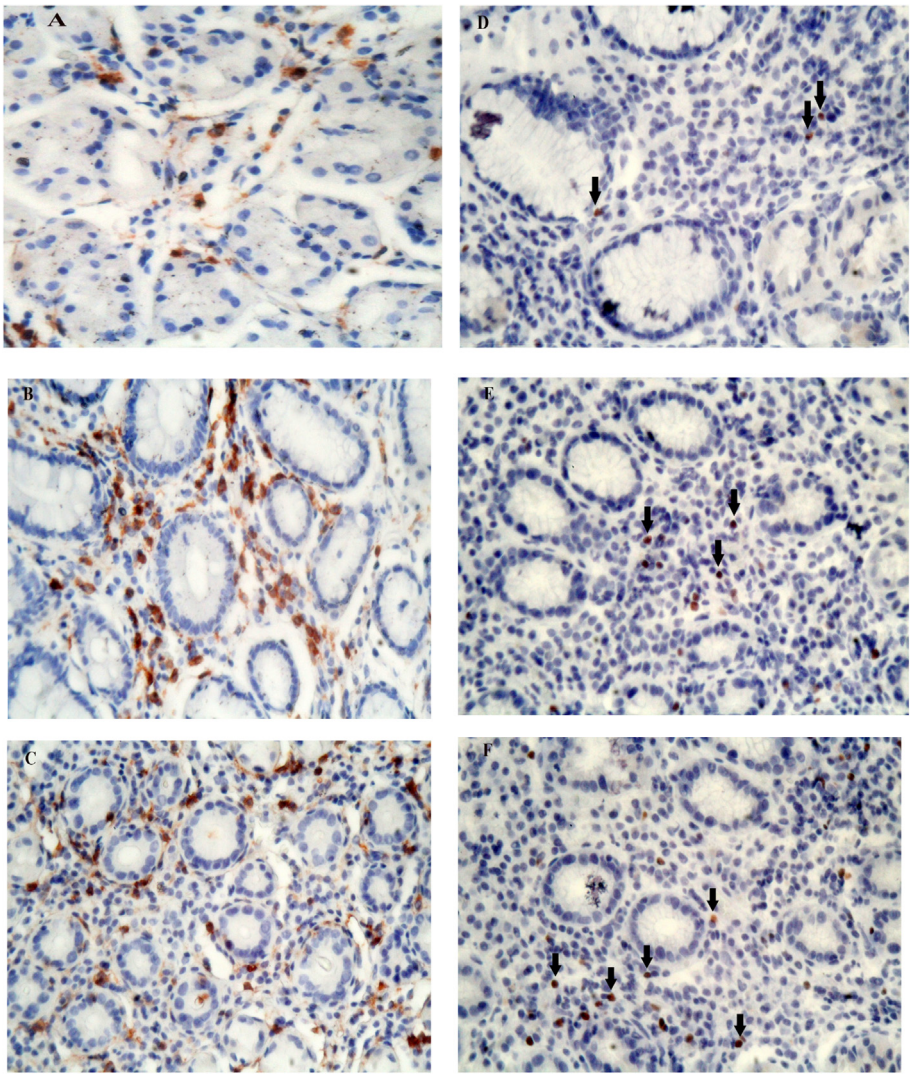

Fig. 1. Immunohistochemical staining of CD4 and Foxp3 T cells in *H. pylori*-negative, gastritis and peptic ulcer biopsies. (original magnification, 400×). Representative immunohistochemical staining of CD4 (left) and Foxp3 (right) in gastric mucosa from A) *H. pylori*-negative individual, B) infected patient with peptic ulcer disease, C) infected patient with gastritis, D) *H. pylori*-negative individual, E) infected patient with peptic ulcer disease and F) infected patient with gastritis. CD4 staining was found on the surface of T lymphocytes, and Foxp3 staining was located in the nucleus of T lymphocytes.

3.3. Relation of $CD4^+$, $Foxp3^+$ T cells and cytokines expression with cagA virulence factor

We also analyzed immunological changes at the mucosal sites with the cytotoxin-associated gene A (CagA) of *H. pylori*. We found that *H. pylori*-infected patients with cagA-positive strains had a significantly higher number of $CD4^+$ T cells compared to the *H. pylori*-infected patients with cagA-negative strains (Fig. 4A; $P = 0.021$). However, no significant correlation in the number of $Foxp3^+$ T cells was noted between *H. pylori*-infected patients with cagA-positive and *H. pylori*-infected patients with cagA-negative (Fig. 4B; $P = 0.133$). In addition, no significant difference was found in the expression of IL-10, TGF-β1, FOXP3, INF-γ and IL-17A mRNA levels between *H. pylori*-infected patients with cagA-positive and *H. pylori*-infected patients with cagA-negative (Fig. 4C–G). Therefore, our data demonstrate lack of association between the mucosal IL-10, TGF-β1, FOXP3, INF-γ and IL-17A mRNA levels with the cagA status of *H. pylori* in our studied patient population.

3.4. Relation of $CD4^+$, $Foxp3^+$ T cells and cytokines expression with oipA virulence factor

We also investigated whether the *H. pylori* outer inflammatory protein (OipA), which is an important virulence factor is associated with observed changes in mucosal biopsies. Interestingly, there was no significant difference in the number of $CD4^+$ T cells seen in the mucosal specimens between *H. pylori*-infected patients with oipA-positive and *H. pylori*-infected patients with oipA-negative (Fig. 5A; $P = 0.184$). However, *H. pylori*-infected patients with oipA-positive had a significantly higher number of $Foxp3^+$ T cells compared with the *H. pylori*-infected patients with oipA-negative (Fig. 5B; $P = 0.005$). In addition, we found that *H. pylori*-infected patients with oipA-positive had a significantly higher expression of IL-10, TGF-β1, FOXP3 INF-γ and IL-17A mRNA levels in comparison to *H. pylori*-infected patients with oipA-negative (Fig. 5C–G: IL-10, $P < 0.0001$, TGF-β1, $P = 0.0277$, FOXP3, $P < 0.0001$, INF-γ, $P = 0.025$, IL-17A, $P = 0.045$). Thus, our data demonstrate the mucosal IL-10, TGF-β1, FOXP3 INF-γ and IL-17A mRNA levels were dependent on the oipA status of *H. pylori*.

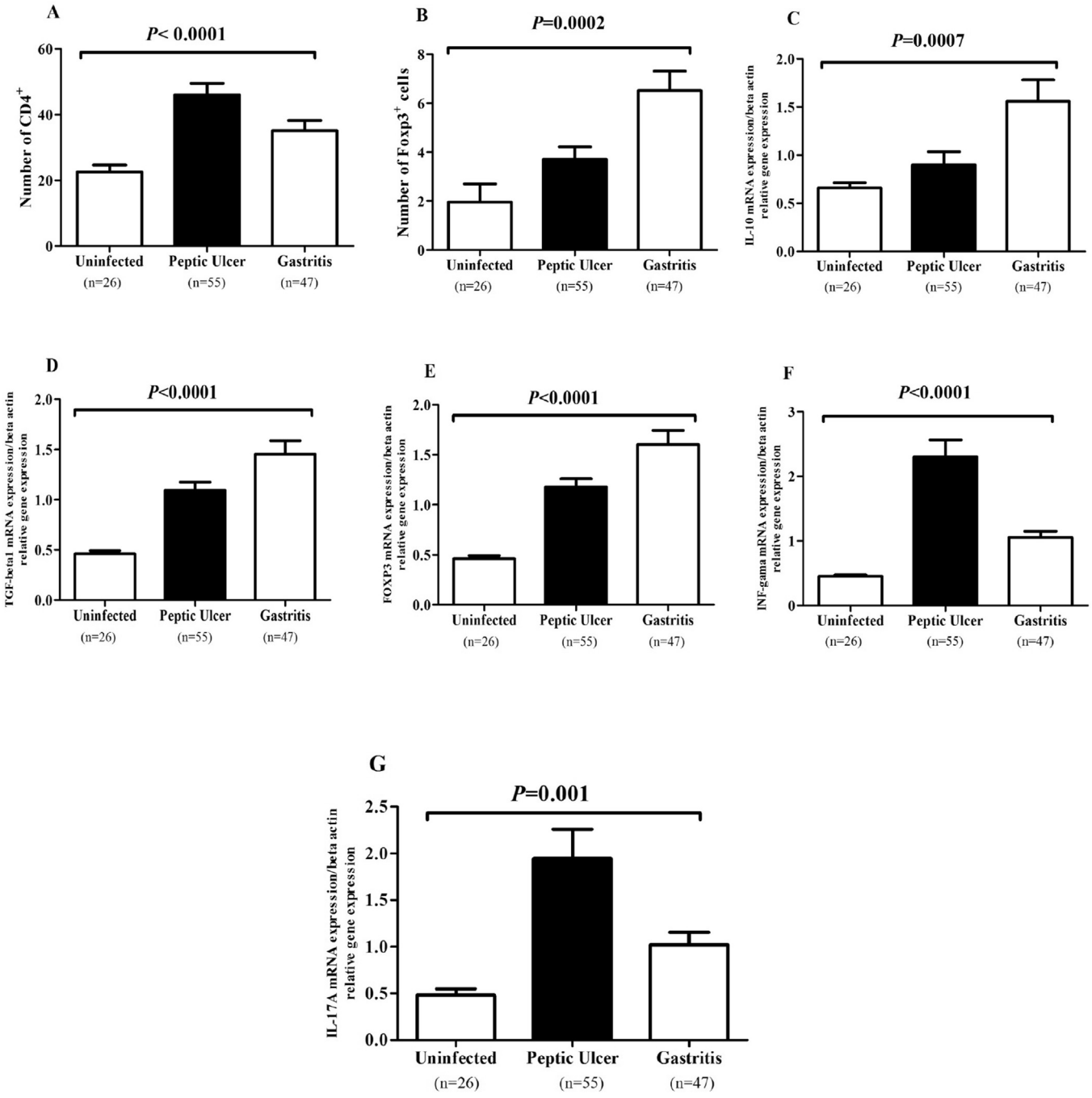

Fig. 2. Column bar graph for the number of CD4$^+$, Foxp3$^+$ T cells and the expression of IL-10, TGF-β1, FOXP3, INF-γ and IL-17A mRNA levels in gastric biopsies of *H. pylori*-uninfected individuals compared with *H. pylori*-infected patients with peptic ulcer or gastritis disease. A) The number of CD4$^+$ T cells and B) The number of Foxp3$^+$ T cells in areas of antral gastric mucosa. RNA was extracted from gastric biopsies of 26 *H. pylori*-uninfected individuals, 55 *H. pylori*-infected patients with peptic ulcer and 47 *H. pylori*-infected patients with gastritis and analyzed for C) IL-10, D) TGF-β1, E) FOXP3, F) INF-γ and G) IL-17A mRNA level by real time-PCR. Levels were normalized to β-actin. *P* values < 0.05 was considered statistically significant using unpaired One-way ANOVA.

3.5. Relationship between virulence genes (vacA, cagA and oipA) and the different gastroduodenal diseases

We studied the relationship between virulence genes (*vacA*, *cagA* and *oipA*) and the different gastroduodenal diseases, between the two groups (patients with peptic ulceration and patients with gastritis). Colonization with *vacA* s1m1 allele and *oipA* positive was significantly higher among gastritis patients in comparison to peptic ulcer patients. Moreover, colonization with *vacA* s1m2 allele was significantly higher among peptic ulcer patients compared with gastritis patients, but when the *cagA* gene was considered, this correlation was not statistically significant (Table 1).

3.6. Relation of CD4$^+$, Foxp3$^+$ T cells and cytokines expression with degree of H. pylori density and chronic inflammation

The number of CD4$^+$ and Foxp3$^+$ T cells was positively associated with *H. pylori* density (Fig. 6A and B: CD4$^+$, $P = 0.0009$ and

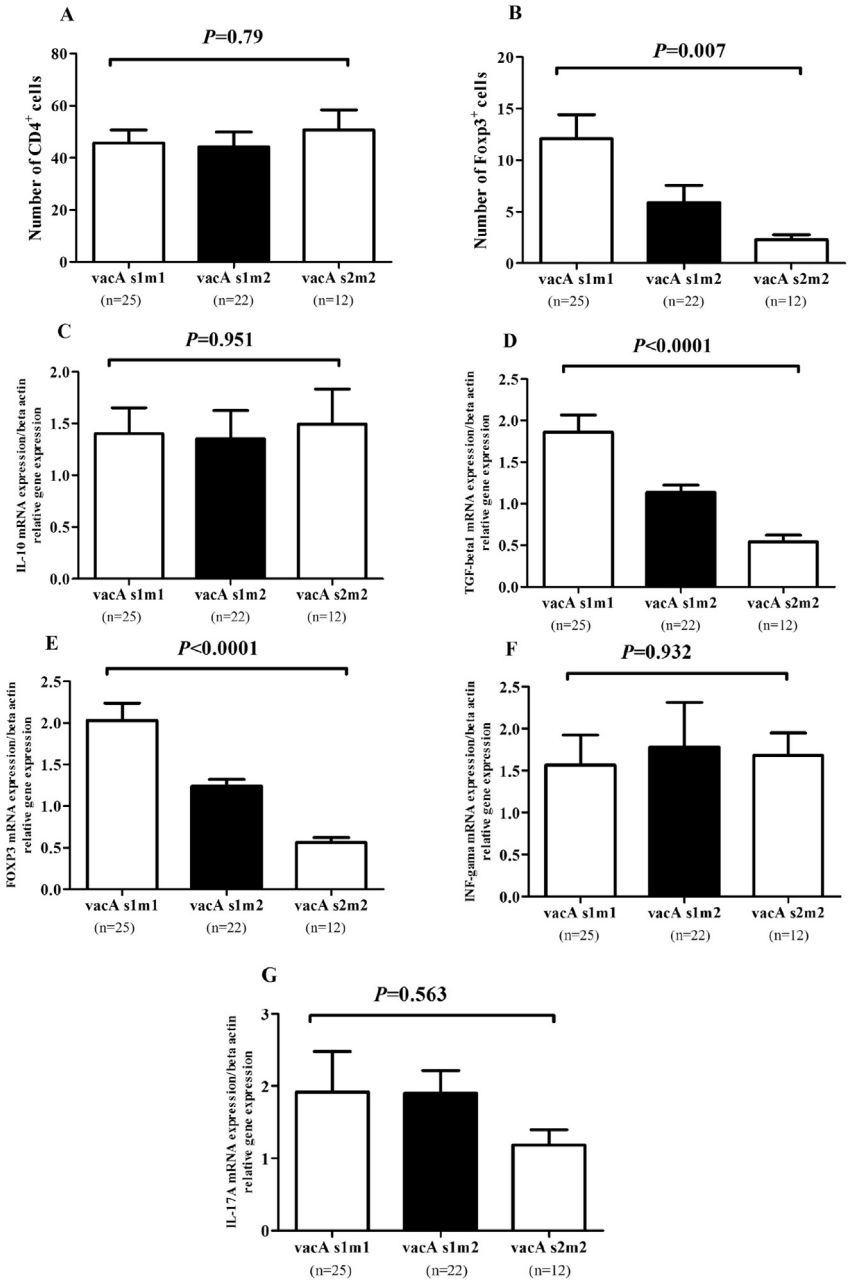

Fig. 3. Column bar graph for the frequencies of CD4$^+$ and Foxp3$^+$ T cells, and the expression of IL-10, TGF-β1, FOXP3 INF-γ and IL-17A mRNA levels according to allelic variants of vacA in patients infected with *H. pylori*. Presence of CD4 and Foxp3 was determined by immunohistochemical staining of mucosal biopsies in two consecutive sections per sample. A) The number of CD4$^+$ T cells, B) The number of Foxp3$^+$ T cells in areas of antral gastric mucosa from patients with *H. pylori* infection. RNA was extracted from gastric biopsies of infected patients; and analyzed C) IL-10, D) TGF-β1, E) FOXP3, F) INF-γ and G) IL-17A mRNA levels by real time-PCR. Levels were normalized to β-actin. P values < 0.05 was considered statistically significant using One-way ANOVA.

Foxp3$^+$, $P = 0.001$). Our results demonstrated that the increase in CD4$^+$ and Foxp3$^+$ T cells is specifically caused by the colonization of *H. pylori*. In addition, the relative expression of IL-10, TGF-β1, FOXP3 and INF-γ were positively associated with *H. pylori* density (Fig. 6C–F: IL-10, $P < 0.0001$, TGF-β1, $P = 0.012$, FOXP3, $P = 0.009$ and INF-γ, $P = 0.022$). The relative expression of IL-I7A was not associated with *H. pylori* density (Fig. 6G, IL-I7A, $P = 0.053$). Furthermore, the number of CD4$^+$ and Foxp3$^+$ T cells were positively correlated with the degree of chronic inflammation (Fig. 6H and I: CD4$^+$, $P < 0.0001$ and Foxp3$^+$, $P = 0.0001$). In addition, the relative expression of IL-10, TGF-β1, FOXP3, INF-γ and IL-I7A mRNA levels were positively associated with the degree of chronic inflammation (Fig. 6J–N: IL-10, $P < 0.0001$, TGF-β1, $P < 0.0001$, FOXP3, $P < 0.0001$, INF-γ, $P = 0.01$ and IL-I7A, $P = 0.002$).

4. Discussion

Most individuals infected with *H. pylori* develop asymptomatic gastritis but some patients develop severe gastric inflammation and peptic ulceration [24]. *H. pylori*-induced gastric mucosal inflammation is mediated by pro- and anti-inflammatory cytokines [25]. In general, studies have shown that this inflammation is exacerbated in patients with a high production of pro-inflammatory cytokines and a low production of anti-

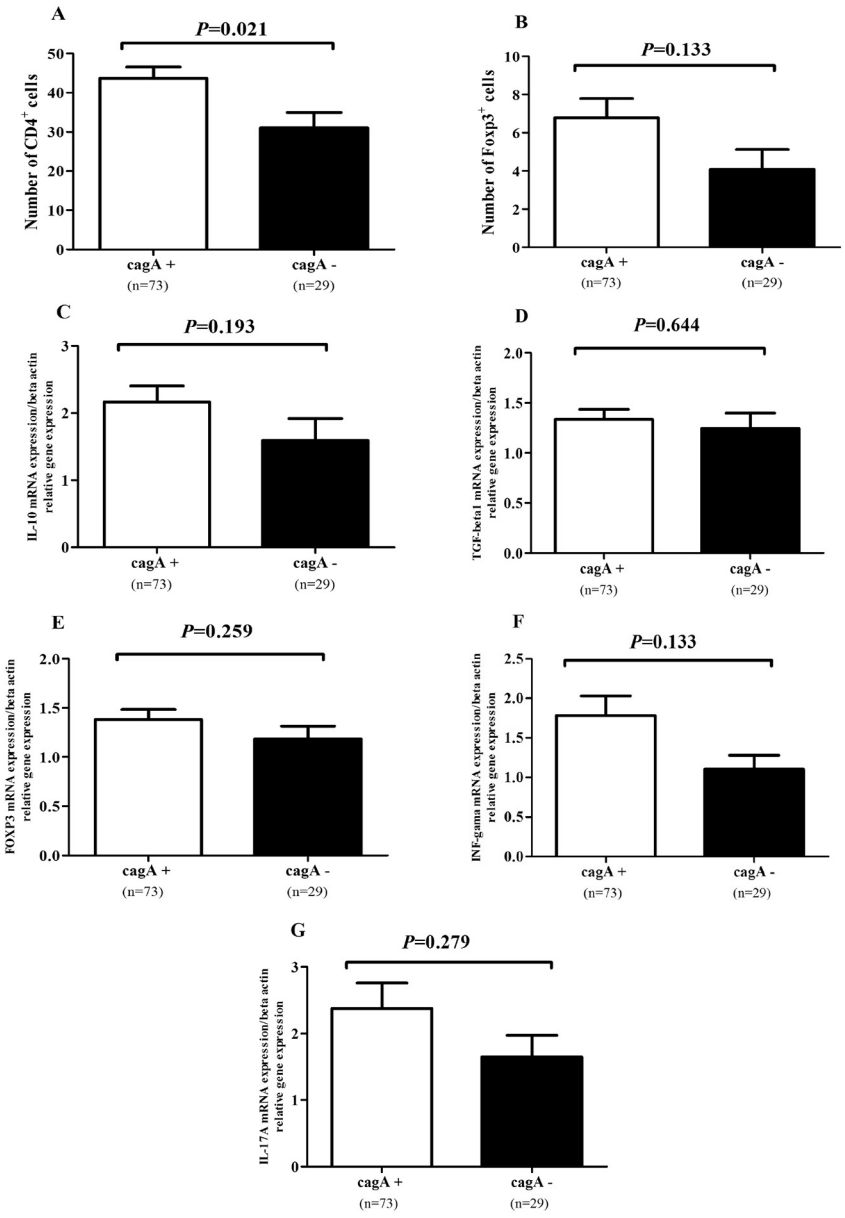

Fig. 4. Column bar graph for the numbers of CD4$^+$ and Foxp3$^+$ T cells, and the expression of IL-10, TGF-β1, FOXP3, INF-γ and IL-17A mRNA level according to cagA virulence factor in patients infected with *H. pylori*. Expression of CD4 and Foxp3 was determined by immunohistochemical staining in two consecutive sections per sample. A) The number of CD4$^+$ T cells, B) The number of Foxp3$^+$ T cells in areas of antral gastric mucosa from patients with *H. pylori* infection. RNA was extracted from gastric biopsies of 73 *H. pylori*-infected patients with cagA-positive and 29 *H. pylori*-infected patients with cagA-negative and analyzed for C) IL-10, D) TGF-β1, E) FOXP3, F) INF-γ and G) IL-17A mRNA level by real time-PCR. Levels were normalized to β-actin. *P* values < 0.05 was considered statistically significant using unpaired Student *t*-test.

inflammatory cytokines, which result in a higher risk of peptic ulcer. Peptic ulcer occurs because of an imbalance between defensive mucosa protective factors and aggressive injurious factors [26]. The results in this study indicated that colonization with s1m2 alleles was significantly higher among peptic ulcer patients than gastritis patients. However, colonization with s1m1 allele and *oipA* positive was significantly higher among gastritis patients in comparison to peptic ulcer ones. This relation was not statistically significant when *cagA* gene was considered. We first confirmed that the number of CD4$^+$ and Foxp3$^+$ T cells in infected patients was significantly higher, compared to uninfected patients. Additionally, the mucosal IL-10, TGF-β1, FOXP3, INF-γ and IL-17A levels were significantly higher in *H. pylori*-positive patients compared with *H. pylori*-negative patients. This study showed that the number of mucosal CD4$^+$ T cells was independent of the different allelic variants of vacA and oipA-virulence factors in patients infected with *H. pylori*, but it was positively correlated with cagA virulence factor in patients infected with *H. pylori.* However, the number of Foxp3$^+$ T cells was dependent on the different allelic variants of vacA and oipA virulence factor in patients infected with *H. pylori*, but it was independent of cagA virulence factor in patients. We found that the number of Foxp3$^+$ T cells, TGF-β1and FOXP3 mRNA levels in infected patients with vacA s1m1-positive was also significantly higher than those observed in infected patients with vacA s1m2-positive and vacA s2m2-positive. We find the mucosal IL-10 mRNA level was independent of the different allelic variants of vacA in infected patients. In support of this finding, many studies have suggested that IL-10 is produced by dendritic cells and gastric

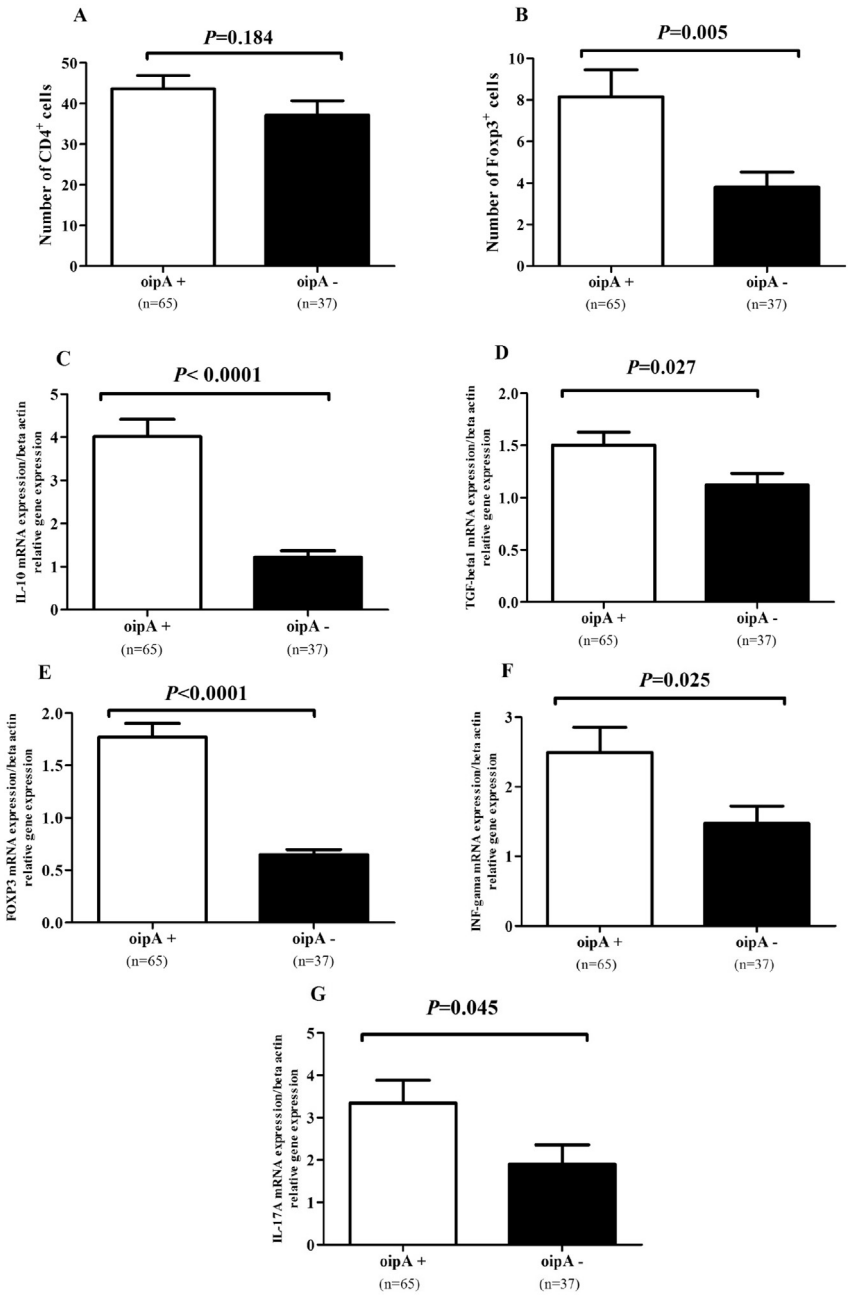

Fig. 5. Column bar graph for the numbers of CD4$^+$ and Foxp3$^+$ T cells, and the expression of IL-10, TGF-β1, FOXP3, INF-γ and IL-17A mRNA levels according to the oipA virulence factor in patients with *H. pylori* infection. Expression of CD4 and Foxp3 was determined by immunohistochemical staining in two consecutive sections per sample. A) The number of CD4$^+$ T cells, B) The number of Foxp3$^+$ T cells in areas of antral gastric mucosa from patients with *H. pylori* infection. RNA was extracted from gastric biopsies of 65 *H. pylori*-infected patients with oipA-positive and 37 *H. pylori*-infected patients with oipA-negative; and analyzed for C) IL-10, D) TGF-β1, E) FOXP3, F) INF-γ and G) IL-17A mRNA levels by real time-PCR. Levels were normalized to β-actin. *P* values < 0.05 was considered statistically significant using unpaired Student *t*-test.

epithelial cells in the gastric mucosa of patients with *H. pylori*. Production of IL-10 by dendritic cells and gastric epithelial cells may interfere the effects of different alleles of the *vacA* gene on the expression of IL-10 production by the Treg cells. In addition the mucosal INF-γ and IL-17A mRNA level was independent of the different allelic variants of vacA in infected patients. We also observed that mucosal IL-10, TGF-β1, FOXP3, INF-γ and IL-17A mRNA levels were dependent on oipA-virulence factor in infected patients however it was independent of cagA-virulence factor in these patients. Our results are in agreement with the study by Oertli et al. [27] which confirmed that both vacA and GGT affect T cell activity in an indirect manner by promoting the preferential differentiation of naive T cells into Tregs. Additionally, the report by Teymournejad et al. has claimed that oipA of *H. pylori* is a DC maturation suppression factor [28]. As a result, *H. pylori* shifts the DC response toward Tregs and away from Th17, which permits persistent infection. Our previous study confirmed that the expression levels of mucosal TGF-β1 were dependent on vacA genotypes, with a positive correlation being reported between secreted vacA s1m1 types and increased mucosal TGF-β1 mRNA and increased mucosal TGF-β1 mRNA levels, thereby contributing to persistent infection [20]. The presence of cagA has been linked to

Table 1
Prevalence of cagA, vacA and oipA status of *H. pylori* among different disease groups.

Genotypes	G[a] [n (%)]	PUD[b] [n (%)]	*P* value[c]
vacA			
s1m1	17 (63.0)	8 (25.0)	0.003
s1m2	4 (14.8)	18 (56.3)	
s2m1	0 (00.0)	0 (00.0)	
s2m2	6 (22.2)	6 (18.8)	
$cagA^+$	39 (70.9)	34 (72.3)	0.873
$cagA^-$	16 (29.1)	13 (27.7)	
$oipA^+$	35 (74.5)	30 (54.5)	0.037
$oipA^-$	12 (25.5)	25 (45.5)	

[a] G: gastritis.
[b] PUD: peptic ulcer diseases.
[c] *P* value was calculated by comparing different clinical outcomes (G and PUD) with respect to *H. pylori* genotype, Chi-square test.

enhanced gastric inflammation [19,29], which is attributed to its potent induction of innate immune responses in epithelial cells. In humans, it is well established that $cagA^+$ *H. pylori* is more potent than cagA *H. pylori* in developing chronic gastritis with higher risks for PUD and GC [30,31]. The study Kido et al. [32] showed that cagA has dual roles in the pathophysiology of *H. pylori*-induced chronic gastritis. The colonization of $cagA^+$ *H. pylori* in the host gastric mucosa is critical for the migration of *H. pylori*-primed $CD4^+$ T cells to the gastric mucosa. In addition, cagA-dependent T-cell priming evokes differentiation of Treg cells. A recently published study indicated an important role for cagA in inducing tolerance [33]. Remarkably, a decline in the ratio of Th1/Th2-derived cytokines was found from asymptomatic gastritis, and other preneoplastic lesions as gastric atrophy or intestinal metaplasia, to gastric adenocarcinoma [34]. This decrease was associated with a concomitant increase in the number of Tregs in peripheral blood and the presence of $cagA^+$ strains [34], indicating that, *H. pylori* favors a Treg-mediated chronic inflammation and consequently the persistence of strains bearing cagA. Several studies have found an epidemiological association between *H. pylori* infection and protection from allergy or asthma [35,36]. Recent data show that a

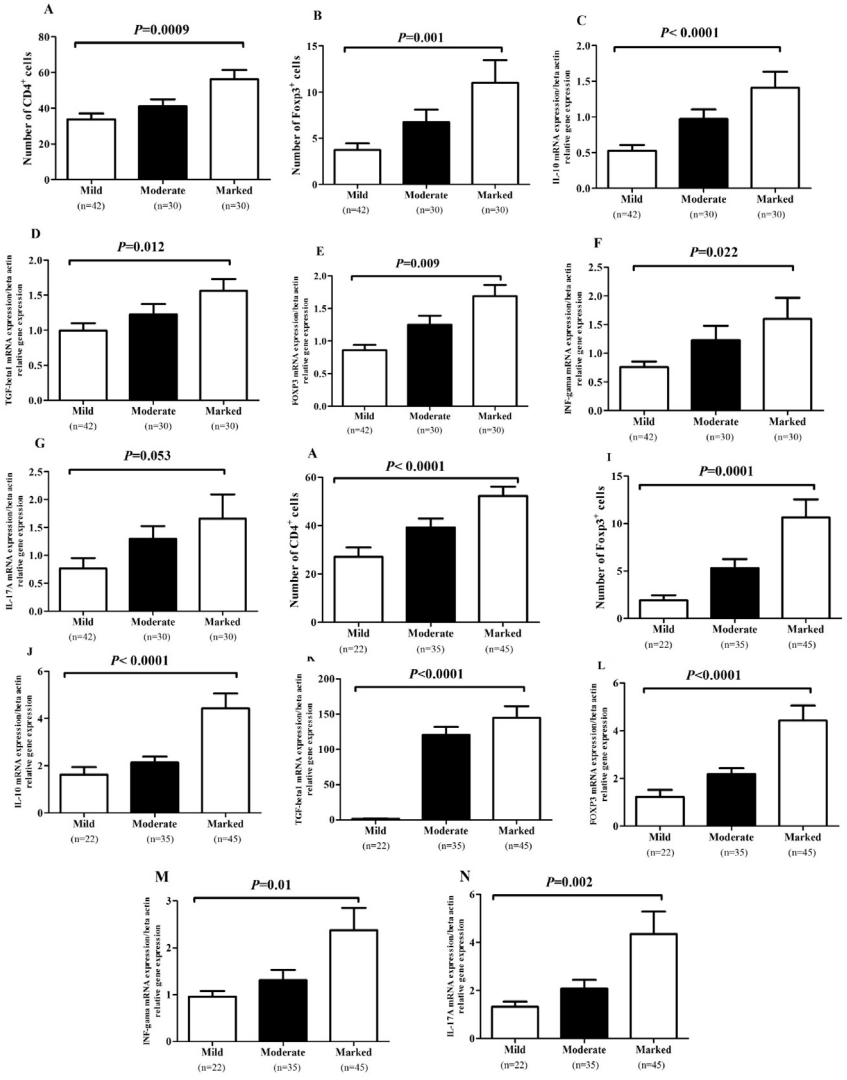

Fig. 6. Column bar graph for the numbers of $CD4^+$ and $Foxp3^+$ cells, and the expression of IL-10, TGF-β1, FOXP3, INF-γ and IL-17A mRNA levels according to *H. pylori* density (graphs A-G) and chronic inflammation (graphs H-N) in infected patients. Expression of CD4 and Foxp3 was determined by immunohistochemical staining in two consecutive sections per sample. A and H) The number of $CD4^+$ T cells, B and I) The number of $Foxp3^+$ T cells in areas of antral gastric mucosa from patients with *H. pylori* infection. RNA was extracted from gastric biopsies of *H. pylori*-infected patients; and analyzed for IL-10, TGF-β1, FOXP3, INF-γ and IL-17A mRNA level by real time-PCR. Levels were normalized to β-actin. P values < 0.05 was considered statistically significant using One-way ANOVA.

stronger protective effect against asthma is associated with *H. pylori* strains expressing the virulence determinant cagA [37]. Such inhibition of allergy and asthma is likely to be mediated by Tregs [38], raising the hypothesis that infection with cagA$^+$ strains may elicit higher levels of Treg response. In contrast with our study, Kao et al. [39] and Zhang et al. [40] demonstrated that *H. pylori*-induced dendritic cells (DCs) skew the Th17/Treg balance toward a Treg-biased response that suppresses Th17 immunity through a cagA and vacA independent, TGF-β and IL-10 dependent mechanism. In support of these findings, Bimczok et al. [41] showed that *H. pylori* was capable of stimulating human gastric DCs to produce IL-10, potentially supplementing Treg suppression of inflammation in the gastric mucosa. Dendritic cells exposed to *H. pylori* failed to induce effector T cell responses of the Th1 and Th17 type *in vitro* and *in vivo*; instead, such DCs preferentially induced the expression of the Tregs specific transcription factor Foxp3, the surface marker of CD25 and the anti-inflammatory cytokine IL-10 in naive T cells [39,42]. This study showed that the number of Foxp3$^+$ T cells and the expression levels of mucosal IL-10, TGF-β1 and FOXP3 in *H. pylori*-infected patients with gastritis were significantly higher than the ones in *H. pylori*-infected patients with peptic ulcer. Also, the number of CD4$^+$ T cells was the lowest in the gastritis patients and increased progressively in the peptic ulcer patients. The expression levels of mucosal IL-17A and INF-γ in *H. pylori*-infected patients with peptic ulcer were significantly higher than the ones in *H. pylori*-infected patients with gastritis. Additionally, the number of CD4$^+$, Foxp3$^+$ T cells and the expression levels of mucosal IL-10, TGF-β1, FOXP3 and INF-γ were positively correlated with the degree of *H. pylori* density and the grade of chronic inflammation. The expression level of mucosal IL-17A was positively correlated with the grade of chronic inflammation but it was independent on the degree of *H. pylori* density. Several studies demonstrated that the number of Tregs is elevated and positively correlated with histological grade of chronic gastritis, atrophic gastritis and adenocarcinoma, but is decreased and negatively correlated with histological grade of intestinal metaplasia [11,15]. Also, Sun et al. [16] demonstrated that chemokine (C-C motif) receptor 2 (CCR2) signaling is essential for DC maturation and function, which are critical for *H. pylori* host immune escape. Their findings show that *H. pylori* infection in CCR2 deficient (CCR2KO) mice exhibits a lower *H. pylori*-specific Treg response resulting in increased gastritis and reduced *H. pylori* colonization. Studies in mouse models have demonstrated that IL-10$^{-/-}$ mice and a strain lacking IL-10 expression in the CD4$^+$ T cell compartment were capable of spontaneously controlling experimental infections [43]. Interestingly, an analogous observation has been reported for human carriers, which both accumulate large numbers of IL-10-producing, *H. pylori*-specific Tregs and are heavily colonized (asymptomatic carriers), or develop gastric ulcers because their Treg response is inadequate [44]. Recent studies in children infected with *H. pylori* have indicated that the number of Tregs is increased in the gastric mucosa of *H. pylori*-infected children compared to adults [45], suggesting Treg participation in the reduced Th1-mediated gastritis and ulceration in the children. In this connection, recent studies also have shown that Treg mediated immune regulation in humans may contribute to *H. pylori* persistence and thus inadequate or lack of Treg responses in adult humans and mice are associated with increased mucosal inflammation during *H. pylori* infection [14,45–47]. In conclusion, induction of Tregs response contributes to peptic ulcer and *H. pylori* colonization. Further studies are required to identify the mechanisms by which *H. pylori* interacts with Tregs and how these cells facilitate infection. A better understanding of the nature, regulation, and function of Treg responses to *H. pylori* may assist in exploring novel and effective immunotherapeutic approaches for gastric diseases induced by this pathogen.

Conflict of interest

The authors have declared that no competing interests exist. All authors have approved this manuscript.

Acknowledgments

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